Biochemical and Biophysical Research Communications 411 (2011) 449–454

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

α-Pinene isolated from *Schinus terebinthifolius* Raddi (Anacardiaceae) induces apoptosis and confers antimetastatic protection in a melanoma model

Alisson L. Matsuo [a,*], Carlos R. Figueiredo [a], Denise C. Arruda [a], Felipe V. Pereira [a], Jorge A. Borin Scutti [a], Mariana H. Massaoka [a], Luiz R. Travassos [a], Patricia Sartorelli [b], João H.G. Lago [b]

[a] Disciplina de Biologia Celular, Departamento de Micro, Imuno e Parasitologia, Universidade Federal de São Paulo, Campus São Paulo, Brazil
[b] Instituto de Ciências Ambientais, Químicas e Farmacêuticas, Universidade Federal de São Paulo, Campus Diadema, Brazil

ARTICLE INFO

Article history:
Received 24 June 2011
Available online 2 July 2011

Keywords:
α-Pinene
Melanoma
Apoptosis

ABSTRACT

Malignant melanoma is one the most aggressive types of cancer and its incidence has gradually increased in the last years, accounting for about 75% of skin cancer deaths. This poor prognosis results from the tumor resistance to conventional drugs mainly by deregulation of apoptotic pathways. The aim of this work was to investigate the cell death mechanism induced by α-pinene and its therapeutic application. Our results demonstrated that α-pinene was able to induce apoptosis evidenced by early disruption of the mitochondrial potential, production of reactive oxygen species, increase in caspase-3 activity, heterochromatin aggregation, DNA fragmentation and exposure of phosphatidyl serine on the cell surface. Most importantly, this molecule was very effective in the treatment of experimental metastatic melanoma reducing the number of lung tumor nodules. This is the first report on the apoptotic and antimetastatic activity of isolated α-pinene.

1. Introduction

Malignant melanoma is one of the leading causes of cancer-related deaths worldwide and its incidence has gradually increased during the last 20 years, accounting for about 75% of skin cancer deaths in United States [1]. Despite the increased progress obtained, combinations of chemotherapy and immunotherapy have not been translated into increased survival. Therefore, development of novel treatment strategies for malignant melanoma is highly encouraged [2]. Natural products from plants, their semisynthetic and synthetic derivatives are important sources of antitumor drugs. According to Cragg and Newman [3], over 50% of the drugs in clinical trials for anti-cancer activity were isolated from natural sources or are related to them. The search for new drugs exhibiting activity on several types of cancer is one of the most interesting subjects in the field of natural products research [4]. The Brazilian biodiversity has become an interesting source for the selection of new chemical compounds that may lead to cancer treatment. Several plant cytotoxic compounds have been found in the Brazilian plants, such as alkaloids [5], terpenoids [6], flavonoids [7], naphthoquinones [8], as well as polyacetylenes [9], which cause cell death by apoptosis.

Apoptosis plays an important physiological role in organ development and tissue homeostasis. It is characterized by alterations in the chromatin structure, DNA fragmentation, exposure of phosphatidyl serine on cell surface, activation of caspases and hyperproduction of reactive oxygen species. In most cases, the cell death is triggered by damage to the outer or inner mitochondrial membrane causing disruption of its potential and release of apoptotic factors. Imperfections in the apoptotic pathway are associated with cancer development and its resistance to chemotherapy. New compounds that overcome cancer cell resistance to apoptosis are advantageous since these tumor cells will be rapidly removed from the tissue by macrophages, preventing local inflammation or an autoimmune response [10,11]. In a previous work we demonstrated the cytotoxic activity on five cancer cell lineages (B16F10-Nex2, A2058, HeLa, MCF-7 and HL-60) of the essential oil and its major component α-pinene derived from *Schinus terebinthifolius* Raddi (Anacardiaceae). Presently, we evaluated the cell death mechanism induced by α-pinene and its therapeutic application on a metastatic melanoma model.

2. Material and methods

2.1. Plant material

Ripe fruits of *S. terebinthifolius* were collected in Ouro Fino at Minas Gerais State, Brazil (349966-W/7536935-N) in March 30th,

* Corresponding author. Address: Department of Microbiology, Immunology and Parasitology, Federal University of São Paulo, São Paulo, SP, Brazil. Fax: +55 1155715877.
E-mail address: alisson@gmail.com (A.L. Matsuo).

0006-291X © 2011 Elsevier Inc. Open access under the Elsevier OA license.
doi:10.1016/j.bbrc.2011.06.176

2008. The botanical identification of the plant was made by Dr. Oriana A. Fávero (Universidade Presbiteriana Mackenzie, São Paulo/SP) and the voucher sample was compared with the register SP272591, deposited at the Herbarium of Instituto de Botânica de São Paulo – SP, Brazil.

2.2. Isolation of α-pinene

The crude essential oil from ripe fruits of *S. terebinthifolius* (400 g) was extracted by steam distillation in a Clevenger type apparatus. After continuous distillation during 4 h, 1.76 g of crude essential oil (yield 0.44%) was obtained after drying with anhydrous Na_2SO_4. Part of this oil (800 mg) was subjected to flash chromatography on Si-gel column eluted with pentane, CH_2Cl_2, and a gradient of CH_2Cl_2–MeOH 95:5 and 9:1 to give 12 fractions which were individually analyzed using gas chromatography (GC) [12]. GC chromatograms of fractions 1–3 showed to be composed by complex mixtures of hydrocarbon derivatives. Thus, this fraction (350 mg) was purified by preparative TLC on Si-gel coated with $AgNO_3$ (10%) eluted with pentane:CH_2Cl_2 1:1 to give 14 mg of pure α-pinene (99% by GC), whose structure was identified by NMR and MS spectral analysis and comparison with literature data [13].

2.3. Mice and cultured cell lines

Eight-week-old male C57BL/6 mice were obtained from the Center for Development of Experimental Models (CEDEME), Federal University of São Paulo (UNIFESP). Animal experiments were carried out according with the UNIFESP Ethics Committee for Animal Experimentation.

The B16F10 murine melanoma cell line is syngeneic in C57Bl/6 mice and was originally obtained from the Ludwig Institute for Cancer Research, São Paulo branch. The subline Nex2 (B16F10-Nex2) was isolated at the Experimental Oncology Unit (UNONEX) and was maintained in complete medium consisting of RPMI 1640, pH 7.2, supplemented with 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid (HEPES), 24 mM sodium bicarbonate, 10% heat-inactivated fetal calf serum (FCS) from Gibco (Minneapolis, MN, USA) and 40 μg/mL gentamicin sulfate (Hipolabor Farmacêutica, Sabará, MG, Brazil) at 37 °C in a humidified atmosphere with 5% CO_2.

2.4. Assessment of mitochondrial $\Delta\Psi_m$

B16F10-Nex2 cells were grown for 24 h in a 12-well plate (1×10^5 cells/well) and incubated with 100 μg/mL of α-pinene at 37 °C for 10 or 30 min. Cells were gently washed with PBS and loaded with 20 nM of tetramethylrhodamine ethyl ester (TMRE, Molecular Probes, OR, USA) for 10 min at 37 °C. Cells were detached with PBS–Trypsin–EDTA and the fluorescence was measured on FACSCalibur (Becton–Dickinson, Franklin Lakes, NJ), using CellQuest software.

2.5. Caspase-3 activity

The enzymatic activity of the caspases induced by α-pinene was assayed using the ApoTarget™ Caspase Colorimetric Protease Assay Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Briefly, the cells were harvested and lysed in the lysis buffer for 10 min on ice bath. The lysate was centrifuged at 10,000*g* for 1 min, and 200 μg of the protein was incubated with 50 μL of reaction buffer and 5 μL of the substrate, at 37 °C for 2 h. The optical density of the reaction mixture was quantified spectrophotometrically at 405 nm wave length.

2.6. DNA degradation assay

Tumor cells (1×10^6) were plated on 24-well plates and incubated for 3 h. Cells were then treated with 100 μg/mL of α-pinene and incubated at 37 °C for 6 h. Centrifuged cells were lysed in TELT buffer (50 mM Tris–HCl pH 8.0, Triton X-100 0.4%, 2.5 mM EDTA pH 9.0 and 2.5 M LiCl) and the lysate was centrifuged for 15 min at 12,000*g*, 4 °C. Buffer-equilibrated phenol was added to the supernatant (1:1, v/v), followed by centrifugation and addition of chloroform (1:1, v/v) to the aqueous phase. After centrifugation (15 min, 12,000*g*, 4 °C), DNA in the aqueous phase was precipitated with sodium acetate 3 M, pH 7.0, and absolute ethanol (1:0.1:2.5, v/v/v) after incubation at −80 °C for 20 min. Precipitated DNA was pelleted and diluted in RNAse-containing water (25 μg/mL). The resulting DNA was electrophoresed in Agarose gel 1% (100 V) and photographed with a digital camera (Kodak, EDAS DC290).

2.7. Annexin V and propidium iodide labeling

B16F10-Nex2 cells were grown for 24 h in a six-well plate (5×10^5 cells/well) and further incubated with α-pinene (100 μg/mL) or medium (control) for 1 or 2 h at 37 °C. The cells were harvested with cold PBS after three washes in the same buffer. Apoptotic/necrotic cells were detected using the ApoScreen Annexin V-FITC kit according to the manufacturer's instructions (Southern Biotechnology, Birmingham, AL) and analyzed on a FACSCalibur equipment (Becton–Dickinson, Franklin Lakes, NJ), using CellQuest software.

2.8. Chromatin condensation analysis

Cells (1×10^4) were cultivated in round coverslips, treated with 100 μg/mL of α-pinene for 20 min, washed with PBS, and fixed for 30 min at room temperature with formaldehyde 2%. Cells were washed with PBS and stained with 2 μM of Hoechst 33342 (Invitrogen) for 10 min. Cells were observed by fluorescence microscopy. Apoptotic cells were detected by chromatin condensation [14].

2.9. Enhanced superoxide anion production

The enhanced superoxide anion production was observed by dihydroethidium (DHE) assay (Invitrogen) as previously described with modifications [15]. B16F10-Nex2 (1×10^4) cells were cultivated in coverslips and treated with 150 μg/mL α-pinene for 1 h. The cells were incubated with 5 μM DHE at 37 °C for 30 min. The conversion of DHE to ethidium by oxidation was observed. As positive control, the cells were treated with 5 mM H_2O_2 for 20 min. Alternatively, a time-lapse conversion of DHE to ethidium was observed incubating 2×10^5 cells in suspension with 5 μM DHE at 37 °C treated or not with 100 μg/mL α-pinene in a 96-well plate. Readings were performed at 488 nm (excitation) and 610 nm (emission) in a Spectramax M2e (Molecular Devices, Silicon Valley, CA). The graphic represents the value obtained by the subtraction of treated cells less the untreated cells.

2.10. Melanoma metastatic assay

In vivo metastatic assays were performed as described by Guimaraes-Ferreira et al. [16]. Male, 7- to 8-week-old C57Bl/6 mice were injected i.v. with 5×10^5 syngeneic B16F10-Nex2 viable cells in 0.1 mL RPMI in each mouse. For antitumor protection experiments, two groups of mice were challenged with tumor cells and treated in the next day with intraperitoneal (i.p.) doses of 100 μL of α-pinene at 10 mg/mL in alternate days or PBS. After 12 days, mice lungs were collected and inspected for metastatic colonization and the melanotic nodules counted at magnification 4×.

3. Results

3.1. Identification of α-pinene

The crude essential oil derived from ripe fruits of *S. terebinthifolius* was analyzed by FID/GC and GC/MS followed by fractionation on SiO_2 and $SiO_2/AgNO_3$ chromatographic columns to afford pure α-pinene. Additionally, the ^1H NMR spectrum (in $CDCl_3$) was recorded and showed singlets of methyl groups at δ 1.26 (H-8), 0.83 (H-9), and 1.66 (H-10), multiplets of allyl hydrogens at δ 2.23/2.16 (H-4), and olephinic hydrogens at δ 5.19 (H-3). These data, associated to ^{13}C NMR data which showed sp^2 carbons at δ 144.6 (C-2) and 116.0 (C-3), methylene groups at δ 31.3 (C-4) and 31.5 (C-7), as well as methyl groups at δ 26.3 (C-8), 20.8 (C-9), and 23.0 (C-10), confirmed the structure of α-pinene [13,17].

3.2. Morphological changes and dissipation of mitochondrial potential by α-pinene

The treatment of B16F10-Nex2 cells with α-pinene induced many morphological alterations as evidenced in Fig. 1A. In 30 min of treatment, cells started to lose its adherence followed by a refringent rounded morphology after 1 h of incubation. On longer (9 h) treatment, cells were opaque with a necrotic appearance.

Some essential oils are able to alter the mitochondrial membrane leading to loss of mitochondrial potential. To evaluate whether α-pinene can promote an early dissipation of mitochondrial potential ($\Delta\Psi_m$), melanoma cells were treated with α-pinene in different times and stained with TMRE (Molecular Probes, OR, USA) for FACS analysis. After 10 min we observed an intense loss of 61.78% in the TMRE fluorescence which was almost abolished after 30 min of treatment (decrease of 93.08%) with α-pinene (Fig. 1B).

3.3. α-Pinene induces apoptosis in melanoma cells

Since the dissipation of $\Delta\Psi_m$ is able to induce apoptosis in cells, we investigated the biochemical and DNA changes caused by α-pinene. An apoptotic hallmark induced by α-pinene was the production of anion superoxide by melanoma cells as inferred from the increased fluorescence of ethidium, the oxidation product of dihydroethidium (DHE) and anion superoxide, observed by microscopy and quantified in a spectrofluorometer (Fig. 2A). Typical apoptotic DNA alterations such as chromatin condensation could be observed in 54% of treated cells by fluorescence microscopy using Hoechst 33258, a DNA staining dye (Fig. 2B) and observation of genomic DNA fragmentation (Fig. 2C, lane 1). Another two important biochemical events during apoptosis were analyzed in treated cells: the activation of caspase-3, a key mediator of apoptosis (Fig. 2D) and the exposure of phosphatidyl serine on cell surface (Fig. 2E) measured by the increase in Annexin V positive cells. In 1 h of treatment, 60% of cells were at early apoptosis stage (Annexin$^+$PI$^-$) and the remaining 40% were at late apoptosis (Annexin$^+$PI$^+$). No primary necrosis (Annexin$^-$PI$^+$) in treated cells was detected.

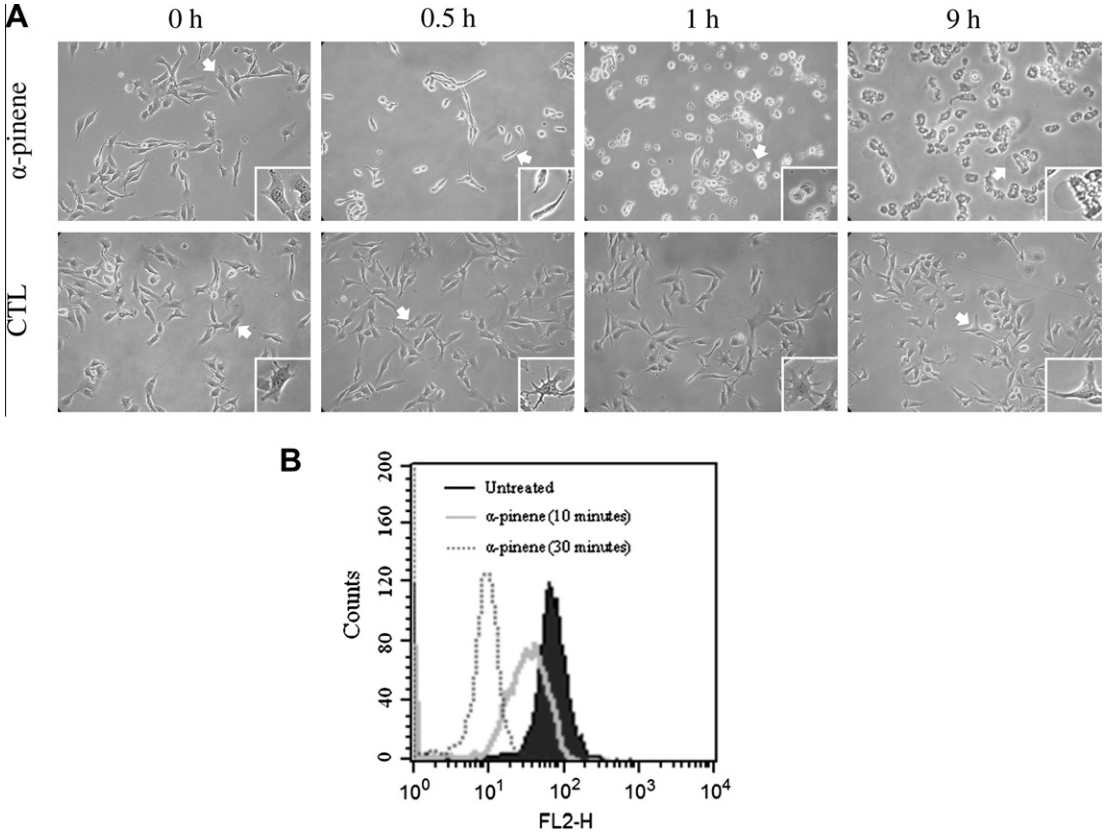

Fig. 1. (A) Cell morphological changes during the treatment with α-pinene. B16F10-Nex2 melanoma cells were incubated with α-pinene and images were captured at different times. After 30 min cells started to lose adherence and became round after 1 h of incubation. In 9 h of treatment, cells were opaque with necrotic appearance. (B) Early dissipation of mitochondrial potential ($\Delta\Psi_m$) by α-pinene. B16F10-Nex2 cells were grown for 24 h in a 12-well plate (1×10^5 cells/well) and incubated with 100 μg/mL of α-pinene at 37 °C for 10 or 30 min. Cells were washed with PBS and loaded with TMRE for 10 min. Cell fluorescence was measured on FACSCalibur. After 10 and 30 min, treated cells lost 61.78% and 93.08% of the fluorescence, respectively.

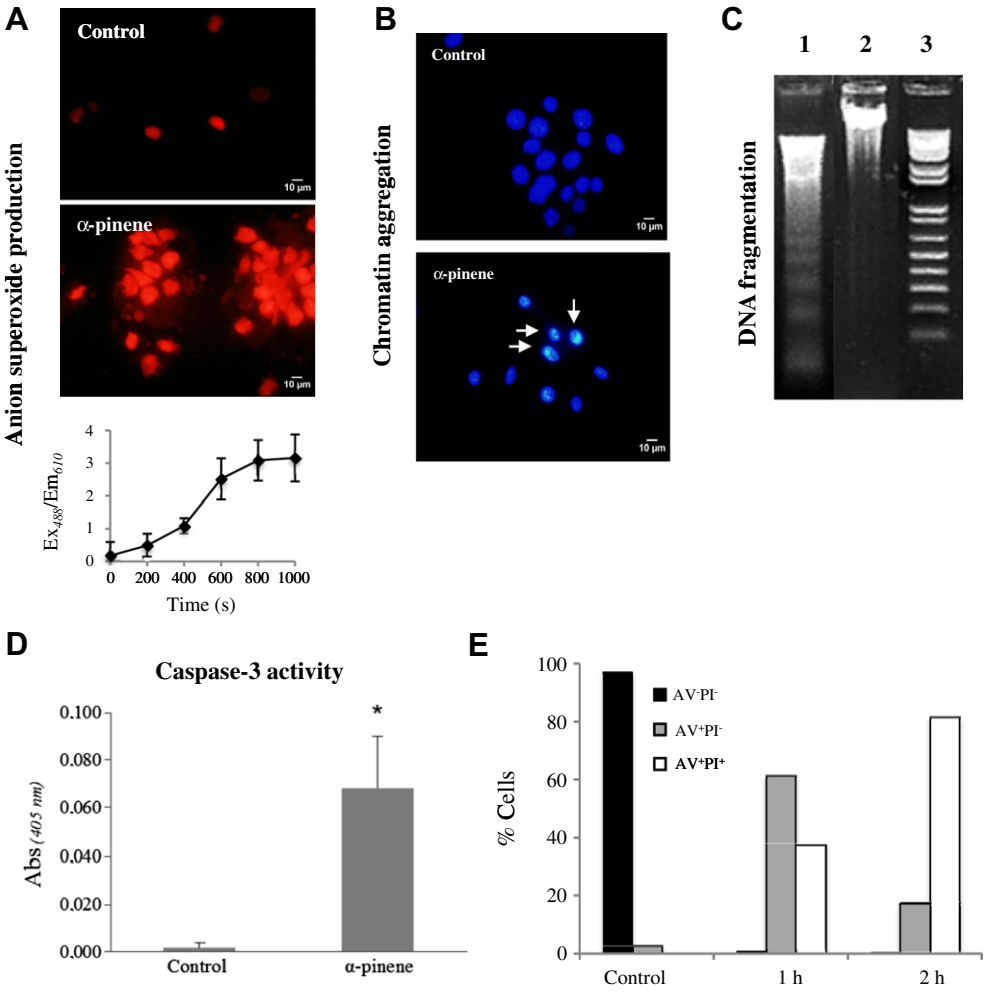

Fig. 2. Apoptosis hallmark features induced by α-pinene. (A) Enhanced superoxide anion production observed by dihydroethidium (DHE). B16F10-Nex 2 cells were treated with 150 μg/mL of α-pinene for 1 h and incubated with DHE. The conversion of DHE to ethidium by oxidation was observed by fluorescence microscopy and quantified in a spectrofluorometer. Magnification 600×. The experiments were performed in triplicates in two independent experiments. (B) Chromatin condensation. Murine melanoma B16F10Nex2.1 (1×10^4) cells were treated with 100 μg/mL of α-pinene for 20 min, stained with Hoechst dye and examined by fluorescence microscopy. About 54% of treated cells presented chromatin condensation indicated by the arrows. Magnification 600×. The experiment was performed in triplicate. (C) Agarose gel electrophoresis of DNA fragmentation induced by α-pinene. DNA was extracted from tumor cells (1×10^6) treated with 100 μg/mL of α-pinene for 6 h and analyzed in Agarose gel. Lane 1: α-pinene; Lane 2: non-treated cells; Lane 3: DNA 1 kb standard. (D) Activation of caspase-3 by α-pinene in melanoma cells. B16F10-Nex2 cells were treated with 100 μg/mL of α-pinene for 1 h and the enzymatic activity of the caspase-3 was determined by a colorimetric assay. Significant differences were determined using the Student's *t*-test ($^*p < 0.01$). Error bars (standard deviation). This assay was performed in triplicate from two independent experiments. (E) Annexin V and PI labeling. B16F10-Nex2 cells were treated with 100 μg/mL of α-pinene for 1 or 2 h and stained with Annexin V-FITC and PI. Analysis was performed using flow cytometry. The values are percentages obtained from two independent experiments.

3.4. In vivo melanoma treatment with α-pinene

In the metastatic experiment, 5×10^5 viable cells of B16F10-Nex2 were injected endovenously in each mouse which was treated in the next day with intraperitoneal (i.p.) doses of 100 μL of α-pinene at 10 mg/mL in alternate days or PBS. This metastatic model was chosen given the aggressive behavior and high metastatic potential of the B16F10 cell line, syngeneic in C57BL6 mice [18]. After 12 days, mice lungs were collected and analyzed for metastatic nodules. Treated mice had a significantly reduced lung colonization ($^*p < 0.01$) showing a potent antimetastatic effect by α-pinene (Fig. 3). In addition, no toxic effect (weight loss or alteration in animal behavior) was observed during the *in vivo* treatment periods.

4. Discussion

Malignant melanoma morbidity and high mortality rate parallels its resistance to conventional chemotherapy. The deregulation of apoptosis, multiple drug resistance and increased DNA repair are factors relevant in melanoma development [19]. Cells that undergo apoptosis are usually phagocytosed by macrophages preventing local inflammation [20]. Essential oils are usually devoid of long-term genotoxic risks and many of them show antimutagenic capacity that can be linked to anticarcinogenic activity. Furthermore, studies have demonstrated that essential oil constituents are very efficient in reducing the tumor volume or tumor cell proliferation by exerting apoptotic effects [21].

Some stimuli to cells can promote changes in the mitochondrion, such as the dissipation of the membrane potential ($\Delta\Psi_m$), decrease of ATP and reduction of translation and transcription, which results in apoptosis and necrosis [22]. In eukaryotic cells, some essential oils can promote depolarization of the mitochondrial membranes by changing their fluidity. Membranes become abnormally permeable resulting in leakage of pro-apoptotic factors such as radicals, cytochrome c, calcium ions and proteins, as in the case of oxidative stress and bioenergetic failure [21]. Since α-pinene induces a rapid loss in the membrane potential but does

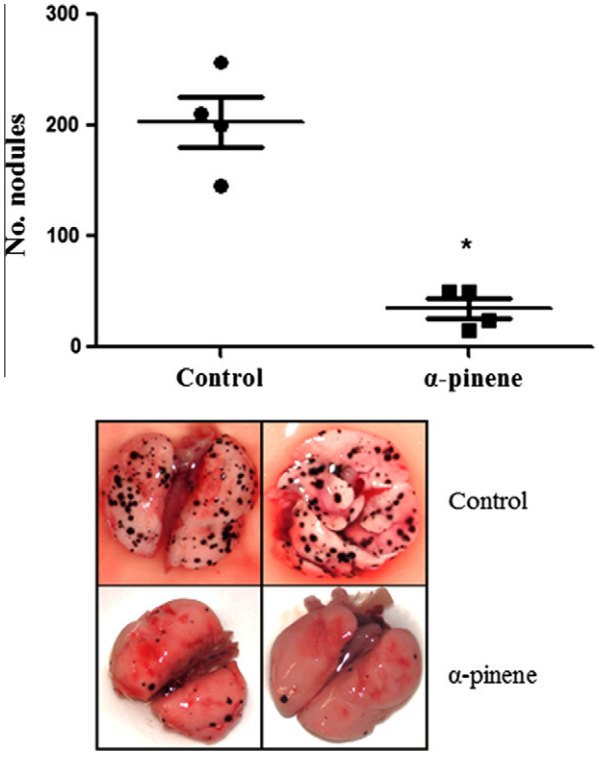

Fig. 3. *In vivo* melanoma treatment with α-pinene. Number of metastatic melanoma nodules present in the lungs of animals treated with 100 μL of α-pinene at 10 mg/mL or the vehicle PBS (control). The compound significantly reduced ($^*p < 0.01$) the number of metastatic nodules as comparing with the untreated control.

not increase the cytoplasmatic calcium on early stages (data not shown) we speculate that this essential oil was able to permeabilize only the outer mitochondrial membrane. Further, cell death was not prevented by cyclosporin A, a potent inhibitor of the inner mitochondrial permeability transition pore (data not shown).

Activation of caspase 3 along with caspase 6 and 7, will finally lead to the apoptotic phenotype [23,24]. This phenotype can be characterized by later phenomena such as heterochromatin aggregation and nuclear DNA fragmentation. Previous works demonstrated that essential oils and other plant derived compounds can induce genomic DNA fragmentation in apoptotic cells [25,26]. It results from the activation of multiple nucleases, but caspase-3 activation is indispensable for heterochromatin aggregation and DNA laddering fragmentation in apoptotic cells [27]. The release of pro-apoptotic factors from the mitochondrial intermembrane space is also associated with generation of reactive oxygen species [28]. In addition to all apoptotic features mentioned above, the plasma membrane phosphatidylserine translocation is typically an early marker of apoptosis [11,29], and is crucial for distinction between apoptosis and necrosis. Several works have shown phosphatidylserine externalization during plant extract, essential oil or specific plant purified compounds treatment indicating their apoptotic potential over tumor cells [30–32].

The Brazilian flora represents a rich source of natural products that could be used as a tool for drug design studies in the development of new therapeutics against several diseases, including cancer. Presently, our results have shown that α-pinene could be a valuable component for the therapy of melanoma, given its great potential to induce apoptosis on cancer cells. Most importantly, mice systemically treated with α-pinene showed a marked reduction in the lung tumor nodules indicating an important activity against metastatic melanoma.

Acknowledgments

The present work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

References

[1] J.S. Lin, M. Eder, S. Weinmann, Behavioral counseling to prevent skin cancer: a systematic review for the US Preventive Services Task Force, Ann. Intern. Med. 154 (2011) 190–201.
[2] B.K. Sharma, V. Manglik, E.G. Elias, Immuno-expression of human melanoma stem cell markers in tissues at different stages of the disease, J. Surg. Res. 163 (2010) e11–e15.
[3] G.M. Cragg, D.J. Newman, Antineoplastic agents from natural sources: achievements and future directions, Expert Opin. Invest. Drugs 9 (2000) 2783–2797.
[4] M.L. de Mesquita, J.E. de Paula, C. Pessoa, M.O. de Moraes, L.V. Costa-Lotufo, R. Grougnet, S. Michel, F. Tillequin, L.S. Espindola, Cytotoxic activity of Brazilian Cerrado plants used in traditional medicine against cancer cell lines, J. Ethnopharmacol. 123 (2009) 439–445.
[5] D.B. da Silva, E.C. Tulli, G.C. Militao, L.V. Costa-Lotufo, C. Pessoa, M.O. de Moraes, S. Albuquerque, J.M. de Siqueira, The antitumoral, trypanocidal and antileishmanial activities of extract and alkaloids isolated from *Duguetia furfuracea*, Phytomedicine 16 (2009) 1059–1063.
[6] G.G. Rocha, M. Simoes, K.A. Lucio, R.R. Oliveira, M.A. Coelho Kaplan, C.R. Gattass, Natural triterpenoids from *Cecropia lyratiloba* are cytotoxic to both sensitive and multidrug resistant leukemia cell lines, Bioorg. Med. Chem. 15 (2007) 7355–7360.
[7] L.H. Cazarolli, L. Zanatta, E.H. Alberton, M.S. Figueiredo, P. Folador, R.G. Damazio, M.G. Pizzolatti, F.R. Silva, Flavonoids: prospective drug candidates, Mini. Rev. Med. Chem. 8 (2008) 1429–1440.
[8] A. Esteves-Souza, K.A. Lucio, A.S. Da Cunha, P.A. Da Cunha, E.L. Silva Lima, C.A. Camara, M.D. Vargas, C.R. Gattass, Antitumoral activity of new polyamine–naphthoquinone conjugates, Oncol. Rep. 20 (2008) 225–231.
[9] H. Buskuhl, R.A. Freitas, F. Delle Monache, A. Barison, F.R. Campos, Y.E. Corilo, M.N. Eberlin, M.W. Biavatti, A new polyacetylene from *Vernonia scorpioides* (Lam.) Pers. (Asteraceae) and its in vitro antitumoral activity, J. Brazil. Chem. Soc. 20 (2009) 1327–1333.
[10] J.K. Brunelle, B. Zhang, Apoptosis assays for quantifying the bioactivity of anticancer drug products, Drug Resist. Updat. 13 (2010) 172–179.
[11] D.V. Krysko, T. Vanden Berghe, K. D'Herde, P. Vandenabeele, Apoptosis and necrosis: detection, discrimination and phagocytosis, Methods 44 (2008) 205–221.
[12] J.H.G. Lago, A.A. Reis, N.F. Roque, Chemical composition from volatile oil of the stem bark of *Guarea macrophylla* Vahl. ssp. tuberculata Vellozo (Meliaceae), Flavour Fragr. J. 17 (2002) 255–257.
[13] K.H. Kubeczka, V. Formácek, Essential Oils Analysis by Capillary Gas Chromatography and Carbon-13 NMR Spectroscopy, second ed., Wiley, 2002.
[14] A.M. Amin, M.C. Lowe, A.A. Ismail, Cytofluorescence localization of adriamycin-binding sites of cardiac tissue, Oncology 40 (1983) 340–343.
[15] M. Mezhybovska, Y. Yudina, A. Abhyankar, A. Sjolander, Beta-catenin is involved in alterations in mitochondrial activity in non-transformed intestinal epithelial and colon cancer cells, Br. J. Cancer 101 (2009) 1596–1605.
[16] C.A. Guimaraes-Ferreira, E.G. Rodrigues, R.A. Mortara, H. Cabral, F.A. Serrano, R. Ribeiro-dos-Santos, L.R. Travassos, Antitumor effects in vitro and in vivo and mechanisms of protection against melanoma B16F10-Nex2 cells by fastuosain, a cysteine proteinase from *Bromelia fastuosa*, Neoplasia 9 (2007) 723–733.
[17] S.G. Lee, Alpha-pinene and myrtenol: complete H-1 NMR assignment, Mag. Res. Chem. 40 (2002) 311–312.
[18] M.L. Ho, Y.S. Hsieh, J.Y. Chen, K.S. Chen, J.J. Chen, W.H. Kuo, S.J. Lin, P.N. Chen, Antimetastatic potentials of *Dioscorea nipponica* on melanoma in vitro and in vivo, Evid. Based Complement Alternat. Med. 2011 (2011) 1–13.
[19] D. Grossman, D.C. Altieri, Drug resistance in melanoma: mechanisms, apoptosis, and new potential therapeutic targets, Cancer Metastasis Rev. 20 (2001) 3–11.
[20] M. Raff, Cell suicide for beginners, Nature 396 (1998) 119–122.
[21] F. Bakkali, S. Averbeck, D. Averbeck, M. Idaomar, Biological effects of essential oils – a review, Food Chem. Toxicol. 46 (2008) 446–475.
[22] B. Mayer, R. Oberbauer, Mitochondrial regulation of apoptosis, News Physiol. Sci. 18 (2003) 89–94.
[23] H. Zou, Y. Li, X. Liu, X. Wang, An APAF-1 cytochrome c multimeric complex is a functional apoptosome that activates procaspase-9, J. Biol. Chem. 274 (1999) 11549–11556.
[24] G. Kroemer, L. Galluzzi, C. Brenner, Mitochondrial membrane permeabilization in cell death, Physiol. Rev. 87 (2007) 99–163.
[25] Y. Yang, Y. Yue, Y. Runwei, Z. Guolin, Cytotoxic, apoptotic and antioxidant activity of the essential oil of Amomum tsao-ko, Bioresour. Technol. 101 (2010) 4205–4211.
[26] S. Kilani, M. Ben Sghaier, I. Limem, I. Bouhlel, J. Boubaker, W. Bhouri, I. Skandrani, A. Neffatti, R. Ben Ammar, M.G. Dijoux-Franca, K. Ghedira, L. Chekir-Ghedira, In vitro evaluation of antibacterial, antioxidant, cytotoxic and

apoptotic activities of the tubers infusion and extracts of *Cyperus rotundus*, Bioresour. Technol. 99 (2008) 9004–9008.
[27] A.G. Porter, R.U. Janicke, Emerging roles of caspase-3 in apoptosis, Cell Death Differ. 6 (1999) 99–104.
[28] J. Cai, D.P. Jones, Superoxide in apoptosis. Mitochondrial generation triggered by cytochrome c loss, J. Biol. Chem. 273 (1998) 11401–11404.
[29] P. Williamson, R.A. Schlegel, Transbilayer phospholipid movement and the clearance of apoptotic cells, Biochim. Biophys. Acta 1585 (2002) 53–63.
[30] D.C. Dartsch, A. Schaefer, S. Boldt, W. Kolch, H. Marquardt, Comparison of anthracycline-induced death of human leukemia cells: programmed cell death versus necrosis, Apoptosis 7 (2002) 537–548.
[31] Y.Y. Tyurina, V.A. Tyurin, Q. Zhao, M. Djukic, P.J. Quinn, B.R. Pitt, V.E. Kagan, Oxidation of phosphatidylserine: a mechanism for plasma membrane phospholipid scrambling during apoptosis?, Biochem Biophys. Res. Commun. 324 (2004) 1059–1064.
[32] G. Seifert, P. Jesse, A. Laengler, T. Reindl, M. Luth, S. Lobitz, G. Henze, A. Prokop, H.N. Lode, Molecular mechanisms of mistletoe plant extract-induced apoptosis in acute lymphoblastic leukemia in vivo and in vitro, Cancer Lett. 264 (2008) 218–228.